

Research Article

Main Variables Affecting a Chemical-Enzymatic Method to Obtain Protein and Amino Acids from Resistant Microalgae

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The development of microalgae uses requires further investigation in cell disruption alternatives to reduce the costs associated to this processing stage. This study aimed to evaluate the main variables affecting an extraction method to obtain protein and amino acids from microalgae. The method was based on a sequential alkaline-enzymatic process, with separate extractions and noncontrolled pH, and was applied to fresh biomass of a resistant species. The processed microalgae were composed of a consortium with *Nannochloropsis* sp. as predominant species. After the optimization of the pH of the alkaline reaction, the effect of the time of the alkaline reaction (30–120 min), the time (30–120 min) and temperature (40–60°C) of the enzymatic reaction, and the biomass concentration (50–150 mg·ml⁻¹), on the extraction yields of protein and free amino nitrogen (FAN) and on the final concentration of protein in the extract, was studied using a response surface methodology. Even though all the variables and some interactions among them had a significant effect, the biomass concentration was the most important factor affecting the overall process. The results showed relevant information about the different options in order to maximize not only the response variables individually but also different combinations of them. Assays with optimized values reached maximum yields of 80.3% and 1.07% of protein (% of total protein) and FAN (% of total biomass), respectively, and a protein concentration in the extract of 15.2 mg·ml⁻¹. The study provided the essential information of an alternative approach to obtain protein and amino acids from fresh biomass of resistant microalgae with a high yield, also opening perspectives for further research in particular aspects.

1. Introduction

Microalgae have a huge benefit as resource to supply the increasing demand of protein and amino acids for food and feed [1], biofertilizers and biostimulants [2], bulk chemicals [3], and bioactives [4]. Nevertheless, economic constraints have hampered the development of these applications, which in general have limited microalgae uses to high-value bioproducts [5–7]. These restrictions are due to mainly the high energetic and economic costs of some stages of the industrial processes, such as harvesting, drying, and cellular disruption [8–10].

With regard to disruption, the selection of the method depends on the cell wall resistance of the species used and

the target molecules, as well as on economic and environmental aspects. Concerning the hardness of the method, it is necessary to reach a compromise between obtaining an acceptable yield and reducing the possible damage of the target bioproduct. In the case of proteins and amino acids, even though it is preferable to use mild methods, sometimes they are not hard enough to disrupt the cell wall and release most of the intracellular molecules, giving rise to low extraction yields, especially from recalcitrant organisms [11]. In particular, microalgae species with resistant cell wall are more suitable to be used in integrated depuration processes to recover residual nutrients, due to their natural resistance against adverse environments. Furthermore, low-cost applications of microalgae require the use of fresh biomass to

avoid the previous costly drying even though microorganisms preserve their cell wall intact, which hinders the bio-products recovering. Disruption techniques such as bead milling and high-pressure homogenization have demonstrated to be very effective and relatively energy-efficient methods [12, 13] though they require specific equipment for which inversion costs are only feasible for some applications [8–10]. In contrast, from an operational point of view, alkaline and/or enzymatic methods could be characterized by their simplicity and therefore applicable to low-cost uses.

Concerning alkaline methods, previous researches have demonstrated that high alkalinities induce cell wall weakening and protein extraction from microalgae [14–16]. Furthermore, enzymatic assisted methods based on proteases enhance the extraction and amino acid production from microalgae biomass [3, 17]. Nevertheless, the combined effect of an alkaline extraction followed by an enzymatic one has not been studied extensively.

This study aimed to evaluate the main variables affecting an extraction method consisting in a sequential alkaline-enzymatic process, with separate extractions and non-pH controlled, to obtain protein and amino acids from fresh biomass of resistant microalgae, avoiding additional disruption pretreatments or drying. The process includes two sequential reactions/extractions: an alkaline reaction for cell disruption and first extraction of proteins, followed by an enzymatic reaction with a commercial protease, for further extraction, and the hydrolysis of the released proteins. The evaluation was performed using a microalgae consortium composed by *Nannochloropsis* sp. as predominant species. Firstly, the optimal pH of the alkaline extraction was established. Subsequently, the main variables of the process were evaluated through a statistical analysis of response surface with a face-centered central composite design (CCD).

2. Materials and Methods

2.1. Microalgae and Cultivation. Microalgae consortium, nonaxenic, was obtained from the Department of Chemical Engineering and Food Technologies, Faculty of Sciences, University of Cádiz. This consortium was isolated in a previous research from a leachate of the landfill “Miramundo-Los Hardales” (Biorreciclaje de Cádiz S.A., Spain). The predominant species was identified by morphologic evaluation, which belongs to the genera of *Nannochloropsis* sp.

Cultures were grown in 5 L borosilicate glass bottles under constant led light ($170 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ average surface irradiance), 12/12 h photoperiod, and room temperature controlled by an air conditioner ($23\text{--}26^\circ\text{C}$). Carbon source, as pure CO_2 , was bubbled by mixing with air into the photobioreactors from the bottom, previously filtered through $0.22 \mu\text{m}$ filters (SLG0510, Merck Millipore, Germany). CO_2 proportion of the mix ($5\text{--}10\% \text{ v.v}^{-1}$) was established on function of the culture pH, between 7.5 and 7.8. Air was introduced at a flow rate of 0.1 vvm. Nutrients were supplied using Combo medium [18], enriched in nitrogen (10 mM) and phosphorus (0.5 mM), providing an excess of these nutrients to maximize protein content in the

biomass. Cultures were carried out in the discontinuous mode for 7 days and a maximum concentration of biomass between 0.7 and 0.8 g.l^{-1} , harvesting in exponential growth phase.

The microalgae biomass was concentrated by centrifugation at $5,000 \text{ g}$ for 15 min and 4°C . To wash the biomass of residual nutrients and salts, this procedure was repeated 2 times resuspending the centrifuged paste in distilled water. The evaluation of the method proposed in this study was performed with the biomass immediately obtained after this procedure (hereafter, referred to as fresh biomass). In addition, dry biomass was produced for analysis of total nitrogen and amino acids content. For these cases, centrifuged paste was frozen at -80°C and freeze-dried (Lyofalfa-85 3.5 kW, Telstar, Spain).

2.2. Experimental Setup

2.2.1. Experimental Procedure. Experiments were performed with suspensions of fresh biomass prepared from centrifuged paste obtained directly from cultures as described above. The required concentrations of the suspensions, calculated in a dry weight basis, were adjusted by dilution of the paste with buffered solutions at different pH depending on the assay, as specified subsequently. Dry weight of the biomass was determined in each experiment by drying in an oven at 105°C for 24 h by the Standard Method 10200 I.5 [19].

The assays were performed in 2 ml centrifugal tubes, in an orbital incubator at 150 rpm, under the temperatures and times specified subsequently in function of the experiment. The first alkaline extraction was performed by incubating the suspension in alkaline buffered solutions at different pH values and 50°C . At the end of the selected reaction times, the supernatants were recovered by centrifugation at $10,000 \times g$ for further analysis (alkaline extracts) and the pellet was kept for the subsequent enzymatic reaction. This pellet was immediately resuspended in 100 mM Tris-HCl at pH 8, diluting till it reaches the initial volume used in the alkaline reaction. Subsequently, the enzymatic reaction was carried out by incubating with Alcalase® 2.5L (Novozymes Spain, S.A., Spain) at different temperatures and times, using an enzyme dosage of 0.2 ml.g^{-1} . The reactions were stopped by inactivating the enzymes at 85°C for 10 min. Subsequently, supernatants were recovered by centrifugation at $10,000 \times g$ for further analysis (enzymatic extracts), and the pellets were discarded. Both alkaline and enzymatic extracts were frozen and stored at -20°C until their analysis.

The buffered solutions used in the assays consisted of pH 8 (100 mM Tris-HCl), pH 10 (50 mM Na_2CO_3), pH 11 (50 mM Na_2CO_3), and pH 12 (50 mM Na_2HPO_4). These pH values correspond to the final values obtained by titration with NaOH or HCl at 25°C . A solution at pH 13.4 was used in high alkaline treatment, obtained by titration with NaOH of a 50 mM Na_2HPO_4 solution. Since the pK_a of the phosphate buffer system ($\text{HPO}_4^{2-}/\text{PO}_4^{3-}$) is 12.33, the maximum theoretical buffering capacity achieved was pH 13.33 [20]. Therefore, the solution at pH 13.4 used in the high alkaline

treatments was slightly above to this maximum buffering capacity. In the case of the enzymatic-buffered solution at pH 8, a higher strength (100 mM) was selected due to the higher acidification expected in the enzymatic reaction as a consequence of the protein hydrolysis.

2.2.2. Effect of the pH of the First Alkaline Reaction. The effect and optimization of the pH of the first alkaline reaction were performed with an assay that consisted of (i) the alkaline reaction at five different pH values using the buffered solutions at pH 8, 10, 11, 12, and 13.4 for 1 h and (ii) the enzymatic reaction with Alcalase® at 0.2 ml·g⁻¹ of dry biomass for 3 h with the buffer solution at pH 8. Both reactions were carried out at 50°C and 150 rpm, using a biomass concentration of 20 mg·ml⁻¹. Blanks were subjected to two sequential extractions at pH 8, without protease in the second extraction, at the same temperature and reaction times. pH 13.4 was selected as optimal of the high alkaline treatment based on preliminary experiments, in which a range of pH from 13.0 to 13.7 was evaluated (data not shown).

2.2.3. Effect of the Main Variables Affecting the Process. The main variables of the sequential process were evaluated using a statistical analysis of response surface with a face-centered CCD: 2⁴ (factorial design), three replications of the centre point, and eight star points ($\alpha=1$). The variables considered were the time of the alkaline reaction (t_a), the time (t_e) and temperature (T_e) of the enzymatic reaction, and the concentration of the biomass (B_c). Factor levels of these variables were selected to cover the range of the optimal expected according to preliminary assays and trying to reduce the impact of the high energetic and economic demanding factors, such as the reaction times (maximum 2 h) and temperatures (maximum 60°C). In the case of the biomass concentration, a maximum of 150 mg·ml⁻¹ was chosen. The range and the levels of the variables are given in Table 1.

Response variables studied were the total yields of the two sequential extractions of protein, expressed as the percentage of the total protein content in the biomass (% of total protein), and free amino nitrogen (FAN), as percentage of the biomass (% of biomass). Additionally, protein content in the extract (mg·ml⁻¹) was considered as the response variable in combined optimizations from the different variables. The design of experiments and the analysis of results were carried out with the statistical software Statgraphics Centurion XVII (Statpoint Technologies, Inc., USA).

The experimental results were fitted with the following second-order polynomial equation:

$$y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} x_i x_j, \quad (1)$$

where y represents the response variable, β_0 is the interception coefficient, β_i , β_{ii} , and β_{ij} are the linear, quadratic, and interaction regression coefficients, respectively, n is the

TABLE 1: Levels of the factors in the experimental design according to the face-centered CCD.

Factor	Unit	Factor level		
		-1	0	1
Time of the alkaline reaction (t_a)	min	30	75	120
Time of the enzymatic reaction (t_e)	min	30	75	120
Temperature of the enzymatic reaction (T_e)	°C	40	50	60
Biomass concentration (B_c)	mg·ml ⁻¹	50	100	150

number of studied variables, and x_i and x_j represent the independent variables.

2.2.4. Validation of the Optimized Conditions. Experiments were carried out using the optimized conditions to confirm and validate models obtained from the analysis of the response surface.

2.3. Analytical Methods

2.3.1. Determination of Total Protein, Total Nitrogen, and Amino Acid Composition. Total protein content of the microalgae consortium was calculated from total nitrogen, multiplied by a nitrogen-to-protein conversion factor obtained from the analysis of the amino acids composition, determined as described below. The nitrogen-to-protein conversion factor (k) was calculated according to Mossé [21], as the average between the upper limit k_A and the lower limit k_P .

Total nitrogen content was determined by elementary analysis (Leco® CHNS 932, Leco Corporation, USA), using the combustion Dumas method [22].

Amino acids composition analysis was performed by high-performance liquid chromatography (HPLC), after 24 h hydrolysis with 6 N HCl at 110°C, and phenylisothiocyanate (PITC) derivatisation according to Henrikson and Meredith [23]. In brief, after the centrifugation of the acid hydrolysate, the supernatant was filtered through an Amicon Ultra-0.5 ml Ultracel-10K centrifugal filter (Merck Millipore, Germany) for deproteinization. The samples were mixed with the derivatizing solution (ethanol:H₂O: triethanolamine:PITC, 7:1:1:1), incubated at room temperature for 30 min, and dried under flowing N₂. The pellet was resuspended in 4 mM sodium phosphate (pH 7.4) and 2% acetonitrile and injected in a HPLC LaChrom Elite® (Hitachi, Japan) system. The separation was performed using a LiChroCART 125-4 column. Amino acids were separated using a linear gradient from 70 mM sodium acetate and 5% acetonitrile buffer (pH 6.55) to acetonitrile/water (50:50).

Ammonia was determined separately by ion chromatography (Metrohm 930 Compact IC Flex, Switzerland) with a pulse amperometric detector with a gold electrode as the working electrode. The elution was carried out in isocratic at a 0.9 ml min⁻¹ flow rate with 1.7 mM HNO₃ and 1.0 mM dipicolonic acid. The separation was performed using a Metrosep C 6-250/4.0 column.

2.3.2. Determination of Soluble Proteins, Peptides, and Free Amino Acids. Soluble protein in alkaline extracts was determined by the Lowry method [24], following the modifications of Bensadoun and Weinstein [25] to remove potential interfering substances. According to the last, proteins of the samples were precipitated with sodium deoxycholate (2%) and trichloroacetic acid (TCA) (24%), by centrifugation at $10,000 \times g$ for 20 min (TCA-Lowry). The standard curve was prepared using bovine serum albumin precipitated following the same procedure. Absorbance was measured at 750 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., USA) in 96-well plates.

The soluble proteinaceous material in enzymatic extracts was determined separately among peptides and free amino acids (and small peptides, which are not detected by the Lowry method). Peptides were determined through the conventional Lowry method, applying a correction factor to take into account potential interfering substances, instead of using TCA precipitation since a large part of them are lost in this precipitation. The correction factor considered was equal to the interference produced in the first alkaline extraction and was calculated dividing the protein determined in the alkaline samples using the TCA-Lowry procedure by the protein determined using conventional Lowry, in each individual experiment.

Free amino acids and small peptides were calculated from the nitrogen determined by FAN analysis using the ninhydrin colorimetric method [26], multiplied by a nitrogen-to-amino acid conversion factor of 5.9. This conversion factor was calculated from amino acids composition analysis (described above) of the alkaline and enzymatic extracts. In the ninhydrin determination, glycine was used as standard and the absorbance was measured at 570 nm using the microplate reader mentioned above. The protein and amino acid weights of the protease used in the enzymatic extractions were subtracted from the results.

2.4. Statistical Analysis of Data. The results were analysed by one-way analysis of variance (ANOVA) with a significance level of p value equal to 0.05, followed by Tukey's multiple comparison test, where applicable. Each experiment was carried out in triplicate, if not stated otherwise. Mean values and standard deviation (SD) are reported.

3. Results and Discussion

3.1. Total Protein. The total protein content of the microalgae consortium was 39.5% of the biomass (dry weight basis). This value was obtained from total nitrogen content in the biomass of 8.93%, multiplied by a nitrogen-to-protein conversion factor of 4.43. The conversion factor is within the range of previously reported values [27, 28].

3.2. Effect of pH of First Alkaline Extraction. Effect of pH of the first alkaline reaction in the sequential process was studied to establish its optimal value and to reduce study variables of subsequent experiments. Figure 1 shows the pH effect in protein extraction yield (proteinaceous material)

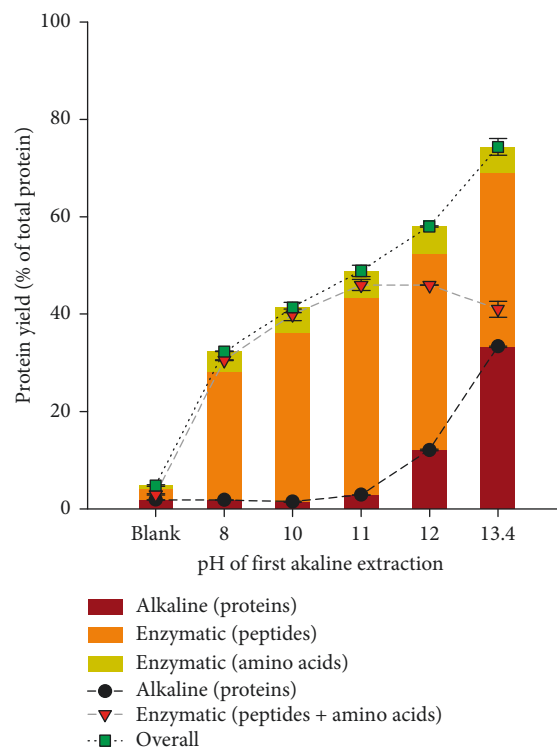


FIGURE 1: Effect of the pH of the first alkaline extraction on protein extraction yield (% of total protein) differing among the two sequential alkaline and enzymatic extractions. The stacked bars represent the partial results obtained in the first alkaline extraction (proteins) and the second enzymatic extraction (differencing among peptides and amino acids). The lines represent the tendency of the same partial results and overall.

differencing among the first alkaline and the second enzymatic sequential extractions, as well as in the overall process (sum of the two extractions).

In relation to the first alkaline extraction, yields were statistically significantly different among the pH values studied (p -value < 0.0001). Multiple comparisons among means showed significantly different yields with respect to control at pH values equal or higher than 11 even though the effect of alkaline treatment was not notable up to pH 12, where the yield achieved 12.1% of total protein, compared to the low value obtained at pH 11 (2.9%). Nevertheless, the highest value by far was obtained at pH 13.4, with an extraction yield of 33.3% of total protein.

In relation to the second enzymatic extraction, results were also statistically significantly different (p value < 0.0001) among the alkalinities tested in the previous alkaline phase. In particular, extraction yield increased from pH 8 (30.5%) to pH 11 (46.0%) of the previous alkaline extraction, with statistically significant differences among them. Above pH 11, enzymatic extraction did not improve due to the fact that most of the proteins were extracted in the previous alkaline phase, being the yield at this pH statistically similar to pH 12 (45.9%) and even higher than at pH 13.4 (41.0%). The increase of yields observed in the enzymatic extraction with the pH of the first alkaline extraction—at least from pH 8 to 11—suggests that higher

alkalinities improve subsequent extraction of proteinaceous material in the second enzymatic step, probably as a result of the cell wall weakening induced by the previous alkaline treatment.

Considering the overall process, the increase of the total yields with the pH of the first alkaline extraction was remarkable, comprising from 32.4% obtained at the low pH value (pH 8) up to 74.3% in the high alkaline treatment (pH 13.4), being all the treatments significantly different among them in multiple comparisons.

In relation to previous studies, using alkaline methods, Gerde et al. [29] obtained similar results at pH 9 and 11 (2%) with *Nannocloropsis* sp., and slightly lower at pH 13 (24.2%), compared with the 33.3% obtained in our study at 13.4, which would be mainly due to a difference of 0.4 in the pH applied. That experiment was performed at 60°C using a non-pH controlled process, over microalgae biomass obtained as frozen paste, which was subsequently defatted and air-dried. Safi et al. [30] obtained higher yields at pH 12, of 31.1 and 33.2% with *Nannocloropsis oculata* and *Chlorella vulgaris*, respectively, at 40°C for 2 h. Those high values with respect to those obtained in our study of 12.1% could be due to the form of initial biomass used, which was freeze-dried biomass of a commercial algae previously purchased as frozen paste. Additionally, Sari et al. [3] achieved higher extraction yields at pH 10 (35%) with *Chlorella fusca* (60°C for 3 h) even though using a pH-controlled process and biomass not directly obtained from cultures. Gerde et al. [29] also improved considerably in their alkaline extraction through a pH-controlled process at pH 11 for 5 h up to 16% and 30%, with defatted and non-defatted biomass, respectively.

It is worth to note that the low extraction achieved in the alkaline reactions between pH 8 and pH 12 at 50°C for 1 h and the second extraction of the blank at 50°C for 3 h, as well as in the enzymatic extraction at pH 8 (30.5%), confirms the recalcitrant character of the algae consortium used. Indeed, Safi et al. [31] reached a similar yield (35%) with *Nannochloropsis gaditana*, a recognized resistant microalgae [32], through an enzymatic reaction using the same protease of this study (Alcalase).

As a result of this assay, pH 13.4 was selected as the optimal pH for the alkaline reaction in the subsequent experiment. The high alkaline treatment performed demonstrated being capable of extracting proteins with a high yield, as well as to weaken the cell wall of recalcitrant microalgae for further enzymatic extraction of the proteinaceous material. In most of the previous researches, high alkaline extraction methods were not studied since the combination of heating and alkaline treatments can modify the protein properties due to denaturation, racemization, and lysinoalanine formation [33], making them unsuitable for some applications. In our study, we selected this very high alkaline treatment with the aim to investigate this alternative for maximizing nitrogen recovery from recalcitrant microalgae using fresh biomass, as a mean to obtain a possible low-cost resource of these bioproducts for many potential applications. For these applications, proteins and amino acids obtained by the method proposed in this study will need further investigation.

3.3. Effect of Main Variables of the Sequential Process. Once established the optimal pH of the alkaline reaction at 13.4, an analysis to evaluate the effect of the main variables involved in the sequential process was performed using a response surface methodology analysis by a face-centered CCD. After the identification of the terms affecting the models, the results were adjusted to fit to second-order equations for both protein and FAN yields, considering the most relevant interactions among the factors. Even though some terms were not statistically significant (p -value < 0.05), they were maintained in the equations to reduce the impact in the R^2 coefficient. The equations with the coefficient obtained were

$$\begin{aligned} \text{protein yield (\% of total protein)} &= 56.3 + 0.325 * t_a - 0.194 \\ &\quad * B_c + 0.133 * T_e \\ &\quad + 0.0251 * t_e - 0.0014 \\ &\quad * t_a^2 - 0.000722 * t_a * B_c \\ &\quad + 0.00102 * t_a * T_e \\ &\quad - 0.00033 * B_c^2 \\ &\quad + 0.000559 * B_c * t_e, \\ \text{FAN yield (\% of total biomass)} &= -1.06 + 0.0035 * t_a \\ &\quad + 0.00555 * B_c + 0.0442 \\ &\quad * T_e - 0.000203 * t_e \\ &\quad - 0.0000215 * t_a^2 \\ &\quad + 0.00000757 * t_a * B_c \\ &\quad - 0.0000343 * B_c^2 \\ &\quad + 0.0000343 * B_c * T_e \\ &\quad + 0.0000281 * B_c * t_e \\ &\quad - 0.000426 * T_e^2. \end{aligned} \quad (2)$$

Both protein and FAN model equations notably suited to the data according to the statistical coefficients obtained. The regression coefficients R^2 were 0.991 and 0.981 for proteins and FAN yields, respectively (adjusted- R^2 = 0.985 and 0.969), indicating that the model explains 99.1% and 98.1% of the dependent variability of the variables. The results obtained in the experiments and the values predicted by the models are displayed in Table 2. The comparison among observed and predicted data shows a notable fit for both protein and FAN models.

Table 3 shows the statistical significance of the variables of the models, revealed by its p values. In the protein model, all the effects studied, as well as the interactions among the biomass concentration with the times of the both alkaline and enzymatic reactions, had a p value lower than 0.05, indicating that their effects were significant with a confidence level of 95.0%. Additionally, the quadratic term of the time of the alkaline reaction was significant. For FAN, all effects studied were also significant and the interaction between the biomass concentration and the time of the

TABLE 2: Experimental design and results of the face-centered CCD.

Run	t_a	B_c	T_e	t_e	Protein (% of total protein)		FAN (% of total biomass)	
					Observed	Predicted	Observed	Predicted
1	-1	-1	-1	-1	61.47	61.36	0.44	0.43
2	+1	-1	-1	-1	71.18	72.14	0.50	0.48
3	-1	-1	-1	+1	66.84	66.13	0.52	0.53
4	+1	-1	-1	+1	79.13	76.91	0.58	0.59
5	0	0	-1	0	57.81	59.17	0.76	0.78
6	-1	+1	-1	-1	35.68	34.85	0.53	0.54
7	+1	+1	-1	-1	40.26	39.13	0.72	0.67
8	-1	+1	-1	+1	43.21	44.65	0.91	0.90
9	+1	+1	-1	+1	47.87	48.93	0.97	1.03
10	0	-1	0	0	73.26	74.07	0.61	0.63
11	-1	0	0	0	55.64	54.21	0.78	0.78
12	+1	0	0	0	61.85	62.66	0.86	0.87
13	0	0	0	-1	56.51	57.63	0.70	0.75
14	0	0	0	+1	65.52	64.92	1.06	0.99
15	0	0	0	0	62.37	61.27	0.87	0.87
16	0	0	0	0	60.39	61.27	0.88	0.87
17	0	0	0	0	60.03	61.27	0.84	0.87
18	0	+1	0	0	48.24	46.83	0.95	0.94
19	-1	-1	+1	-1	65.72	64.64	0.49	0.49
20	+1	-1	+1	-1	77.85	77.25	0.56	0.55
21	-1	-1	+1	+1	67.36	69.41	0.59	0.60
22	+1	-1	+1	+1	81.14	82.03	0.67	0.66
23	0	0	+1	0	65.67	63.37	0.88	0.88
24	-1	+1	+1	-1	36.61	38.13	0.64	0.67
25	+1	+1	+1	-1	43.52	44.24	0.81	0.80
26	-1	+1	+1	+1	48.79	47.93	1.05	1.04
27	+1	+1	+1	+1	54.55	54.05	1.15	1.16

TABLE 3: Regression coefficients, F values, and p values of the variables and interactions in the equation models.

Term	Coefficient	F value	p value
Protein			
Intercept	56.3	—	—
A: t_a	0.325	138.21	<0.0001 ^a
B: B_c	-0.194	1437.9	<0.0001 ^a
C: T_e	0.133	34.13	<0.0001 ^a
D: t_e	0.0251	102.9	<0.0001 ^a
$t_a \times t_a$	-0.0014	11.54	0.0034 ^a
$t_a \times B_c$	-0.000722	18.19	0.0005 ^a
$t_a \times T_e$	0.00102	1.45	0.2448
$B_c \times B_c$	-0.00033	0.98	0.3371
$B_c \times t_e$	0.000559	10.89	0.0042 ^a
FAN			
Intercept	-1.06	—	—
A: t_a	0.0035	33.32	<0.0001 ^a
B: B_c	0.00555	362.94	<0.0001 ^a
C: T_e	0.0442	39.25	<0.0001 ^a
D: t_e	-0.000203	209.15	<0.0001 ^a
$t_a \times t_a$	-0.0000215	4.49	0.0501
$t_a \times B_c$	0.00000757	3.94	0.0647
$B_c \times B_c$	-0.0000343	17.4	0.0007 ^a
$B_c \times T_e$	0.0000343	3.98	0.0635
$B_c \times t_e$	0.0000281	54	<0.0001 ^a
$T_e \times T_e$	-0.000426	4.3	0.0546

^aStatistically significant at 95% confidence level.

enzymatic reaction, as well as the quadratic term of the biomass concentration, was also significant.

Pareto diagrams showed in Figure 2 represent graphically the standardized effects of the variables and interactions. In both protein and FAN models, the concentration of the biomass was the main variable affecting the global process. Nevertheless, in protein yield, the effect of the biomass concentration was negative and in FAN was positive.

In the case of proteins, the negative effect of biomass concentration was probably caused by the dilution of the alkaline medium by the biomass volume at the higher biomass concentrations. This would lead to a significant drop of the pH at $t=0$ (non-pH controlled process). Indeed, the initial pH 13.4 used in the buffer declined to pH 13.0, 12.8, and 12.5 at initial time ($t=0$) with 50, 100, and 150 mg·ml⁻¹ of initial biomass, respectively. This affects negatively both the first alkaline extraction and the subsequent enzymatic extraction, due to the lower weakening of the cell wall produced. Additionally, pH continues diminishing up to the final reaction to reach pH of 12.0, 11.0, and 10.5 in 50, 100, and 150 mg·ml⁻¹, respectively. This reduction with the time is probably due to the buffering properties of the proteins released, as previously have been reported. Gerde et al. [29] found, using an alkaline extraction at pH 11, a significant decrease of the pH, probably due to the protein dissolution and its buffering effect. In their case, this reduction was mainly due to the buffering effect since the concentration of the biomass suspension used was very low (1%). Indeed, in that study, at pH 13 and 1% of biomass suspension, the extracted proteins were not enough to reduce the pH.

Concerning the other variables analysed, the time of the alkaline reaction had a positive effect in the overall protein extraction yield mainly due to its effect in the extraction in the first alkaline reaction. Furthermore, the time and temperature of the second enzymatic reaction had a positive effect since these variables improve enzymatic action of the protease used in the second extraction.

In contrast, in the case of the FAN, increments in the initial biomass concentration had a positive effect because they enhance the action of protease as a consequence of the effect of a higher substrate concentration at the same enzyme dosage. Moreover, the time and temperature of the enzymatic reaction had a significantly positive effect. It is worth to note that, in this case, the time of the alkaline reaction had a positive and significant effect, which suggests that the first alkaline reaction not only affects the first extraction directly but also facilitates subsequently the action of the protease in the second extraction due to the weakening of the cell wall.

The first-order surfaces for both models are shown in Figure 3. In the protein model, the surface shows that the extraction yield decreases notably with biomass concentration, following a next to linear tendency. In contrast, the yield increased with the time of the alkaline reaction, showing a more marked curvature at higher biomass

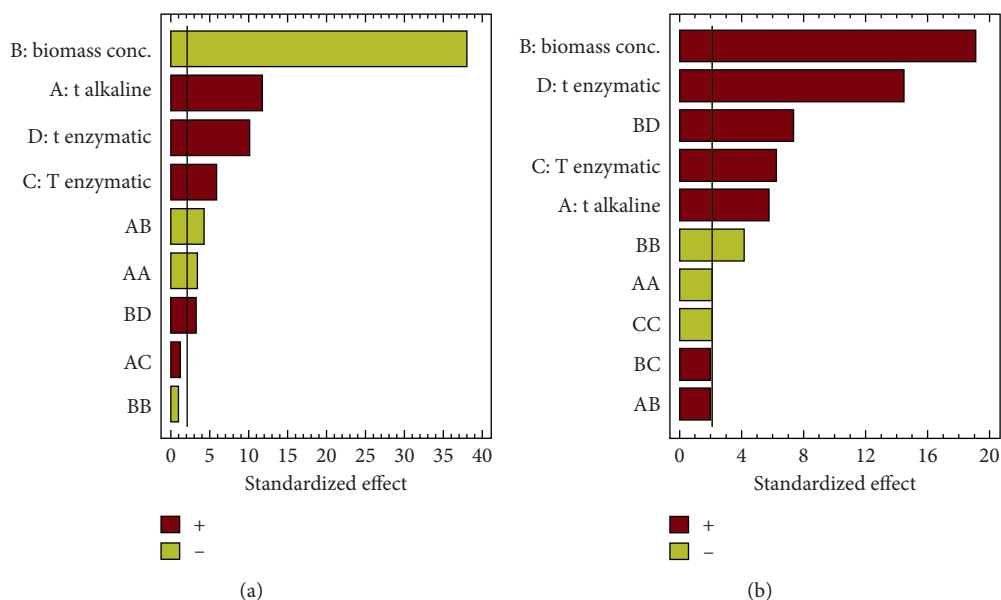


FIGURE 2: Pareto diagrams showing the standardized effects of the variables and interactions on the total extraction yield of (a) protein (% of total protein) and (b) FAN (% of total biomass).

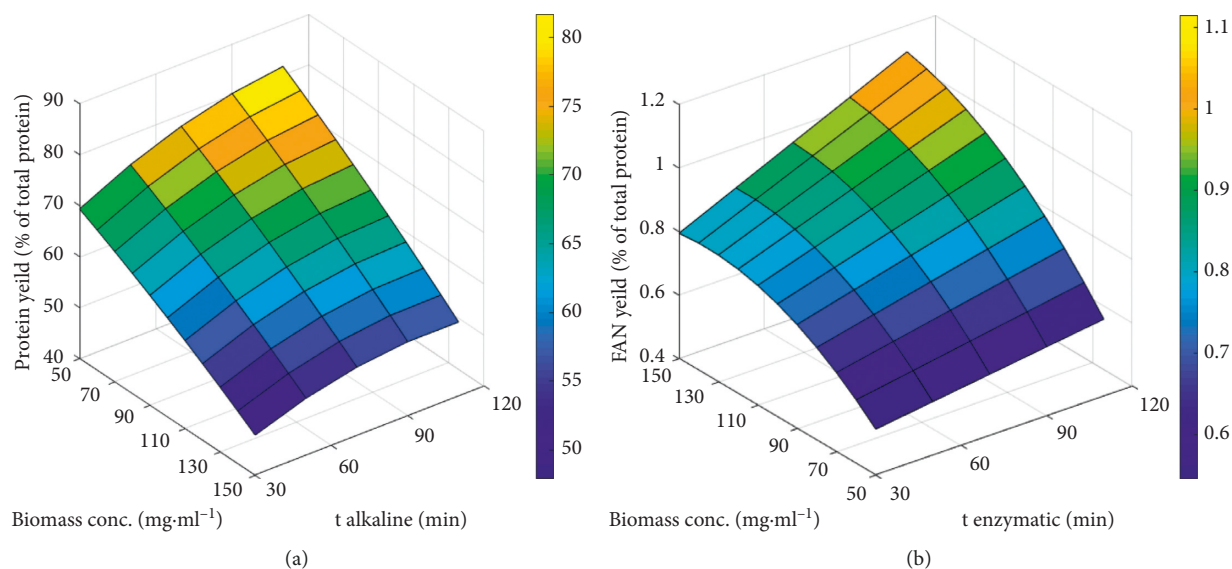


FIGURE 3: Response surface diagrams showing (a) the effect of the biomass concentration and the time of the alkaline reaction on the protein yield (% of total protein) ($t_e = 120$ min and $T_e = 60^\circ\text{C}$) and (b) the effect of the biomass concentration and the time of the enzymatic reaction on FAN yield (% of total biomass) ($t_a = 120$ min and $T_e = 60^\circ\text{C}$).

concentrations, which indicates that, in higher concentrations, the application of more time in the alkaline reaction is not enough to reach a substantial improvement of the yield. Finally, the maximum protein extraction yield was reached at the minimum biomass concentration ($50\text{ mg}\cdot\text{ml}^{-1}$) and maximum alkaline time (120 min). Furthermore, in the case of FAN model, yield increased with both the biomass concentration and the time of the enzymatic reaction. Nevertheless, while the model showed a curvature for the biomass concentration, the tendency for the time enzymatic reaction was linear in the range studied. Indeed, the maximum FAN

extraction yield was achieved at the maximum biomass concentration ($150\text{ mg}\cdot\text{ml}^{-1}$) and maximum enzymatic time (120 min).

3.4. Optimization and Validation of the Models. Table 4 shows the optimal values obtained from the models to maximize the response variables. In addition to the protein and FAN models, it includes the optimal of combined response variables. The concentration of the protein in the final extract has also been included in the combined analysis due to its importance in an industrial point of view (model

TABLE 4: Optimal values of the factors to maximize the different response variables.

Term	Response variable*					
	<i>P</i>	<i>F</i>	<i>P + F</i>	[<i>P</i>]	<i>P + [P]</i>	All
Time of the alkaline reaction (t_a) (min)	120.0	108.0	106.0	111.3	111.2	106.7
Time of the enzymatic reaction (t_e) (min)	120.0	120.0	120.0	120.0	120.0	120.0
Temperature of the enzymatic reaction (T_e) (°C)	60.0	58.0	60.0	60.0	60.0	60.0
Biomass concentration (B_c) (mg·ml ⁻¹)	50.0	150.0	102.1	150.0	104.0	121.3
Predicted response	<i>P</i>	82.0	54.1	68.3	54.4	67.8
	<i>F</i>	0.66	1.16	1.02	1.17	1.02
	[<i>P</i>]	8.9	15.3	14.4	16.2	14.5

**P*: protein yield; *F*: FAN yield; +: combined optimization; [*P*] = protein concentration in the final extract; All: combined optimization of *P + F + [P]*.

not shown, with a $R^2 = 0.995$). In all cases, the time and the temperature of the enzymatic extraction obtained the same or close optimal values, 120 min and 60°C, respectively, which corresponded to the maximum values established in the experimental design. The time of the alkaline reaction varied among the response variables, with a maximum of 120.0 min for maximizing the protein extraction yield. The optimal of initial biomass concentration range from the minimum experimental value used (50 mg·ml⁻¹) to the maximum (150 mg·ml⁻¹) for maximizing protein and FAN extraction yields, respectively. Intermediate biomass concentrations of around 100 mg·ml⁻¹ and 120 mg·ml⁻¹ improve yields in combined response variables.

To perform the validation of the models, experiments using the optimal values to maximize the protein yield, except the biomass concentration, were selected ($t_a = 120$ min; $t_e = 120$ min; $T_e = 60^\circ\text{C}$). In the case of the biomass concentration, 50, 100, and 120 mg·ml⁻¹ were chosen because these values were optimal in different combined variables optimizations (Table 4). 150 mg·ml⁻¹ was not selected because this concentration reached a very low yield (54.4%), which was not sufficiently compensated with the values obtained by the other response variables.

As a result of these experiments, total extraction yields of protein were 80.3, 67.5, and 63.5% in 50, 100, and 120 mg·ml⁻¹, respectively (Figure 4), compared with the predicted by the model of 82.0, 68.9, and 63.1%, which represent a very good fit since in the worst case, the error was of 2.1%. In the case of FAN, extraction yields reached 0.64, 0.99, and 1.07% in 50, 100, and 120 mg·ml⁻¹, respectively (Figure 4), which were close to the model predicted values of 0.66, 1.00, and 1.08%. The concentrations of protein in the final extracts reached 7.9, 13.9, and 15.2 mg·ml⁻¹ in 50, 100, and 120 mg·ml⁻¹, respectively.

The protein extraction yields obtained in this study through two sequential extractions are higher than those of the previously reported researches with resistant microalgae species using different extraction methods, i.e., 4.4% by pulsed electric fields [34], 21% by bead milling [35], 30% by alkaline extraction [29], 35% by enzymatic treatment [31], and 50% by high-pressure homogenization [30]. Sari et al. [3] also obtained a high yield of 69% by an enzymatic

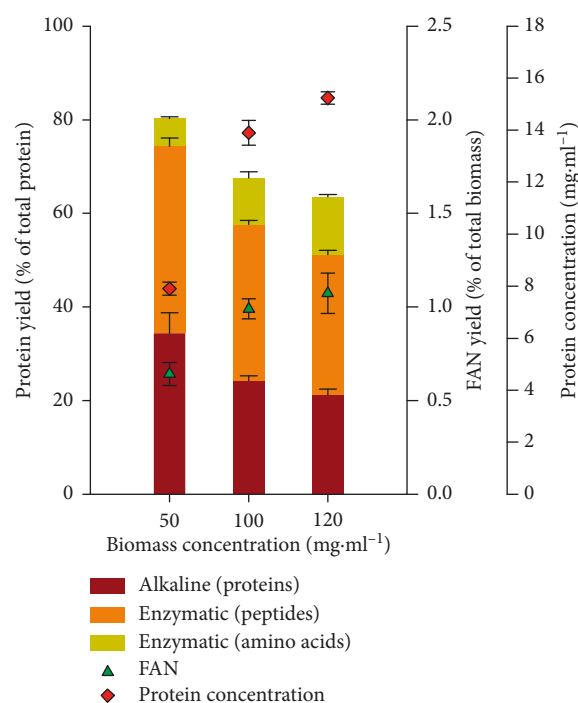


FIGURE 4: Effect of three biomass concentrations on the extraction yields of protein (% of total protein) and FAN (% of total biomass) and on the protein concentration in the final extract (mg·ml⁻¹), differing among first alkaline and second enzymatic extractions.

assisted extraction with proteases, even though using a pH-controlled process.

An electrophoresis using SDS-PAGE was performed to qualitatively evaluate the proteins and peptides existing in the two extracts and to determine their molecular weights (Figure S1). In the alkaline extract, proteins comprised all the range of molecular weights, including high-weight proteins around 250 kDa, in accordance with previous studies using alkaline solutions, i.e., at pH 11 [29] and pH 12 [36]. In particular, the band around 17 kDa showed a slight lower dye intensity even though below this molecular weight, the intensity notably increased. This higher intensity between 11 and 17 kDa is similar to that obtained in the mentioned studies using lower

pH; therefore, it could not be associated to a significant higher degradation of the proteins as a consequence of the high alkaline treatment. In contrast, in the enzymatic extract, the presence of proteins was practically inexistent, except in a slight band with a higher intensity around 11 kDa, due to the hydrolysis to small peptides and amino acids with lower molecular weights. This is in accordance with a previous research using a protease for extracting proteins from microalgae, in which the bulk of the peptides obtained comprised molecular weights lower than 12 kDa [37].

The quality of the different extracts should be also further investigated depending on the specific application. The first alkaline treatment can affect the protein properties by denaturation, racemization, and lysinoalanine formation, which could hinder the development of some applications depending on the degree of the affectation, such as emulsifying or foaming substances. Peptides from the enzymatic extract are especially interesting for benefits such as use as bioactive molecules, fertilizers, and culture medium of microorganisms, among others.

4. Conclusions

This study provides an alternative approach to obtain protein and amino acids from fresh biomass of resistant microalgae with a high yield. The response surface methodology used has revealed the main effects of the variables affecting the sequential alkaline-enzymatic process and to build models to predict the response of the method. The different existing options identified to maximize not only the response variables individually but also different combination of them provide a high versatility to the method. Indeed, because the biomass concentration is the most important factor affecting the overall process, different results can be obtained by only modifying this variable. A maximum protein yield of 80.3% is obtained using a concentration of biomass of 50 mg·mL⁻¹, at the expense of FAN yield (0.64%) or protein concentration in the final extract (7.9 mg·mL⁻¹). In an intermediate value of biomass concentration (100 mg·mL⁻¹), the yields reach 67.5% and 0.99% of protein and FAN, respectively, and the concentration of protein in the final extract achieves 13.9 mg·mL⁻¹. Furthermore, this study has revealed other factors that could be further investigated such as using a sequential alkaline-enzymatic method with a single final extraction, testing the process over other recalcitrant microalgae species, and the control of the pH of the process, as well as the quality and suitability of the products obtained through the method proposed for different applications.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Figure S1. SDS-PAGE of the different extracts obtained by the sequential alkaline-enzymatic process, with separate extractions and non-controlled pH, using fresh biomass at a concentration of 100 mg·mL⁻¹. (*Supplementary Materials*)

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